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Preparation of niosomes as an ocular delivery system for naltrexone hydrochloride: Physicochemical characterization

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Recent reports have demonstrated that topical and systemic application of naltrexone markedly improves the characteristic signs of diabetic keratopathy; most notably, impaired corneal sensation and delayed wound repair. The aim of this study was to prepare and characterise non-ionic surfactant vesicles (niosomes) for the ocular drug delivery of naltrexone hydrochloride. The niosomes were prepared using the thin film hydration method and characterised using polarized light microscopy, cryo-scanning electron microscopy (Cryo-SEM), percent drug entrapment efficiency (EE %), laser light diffraction and differential scanning calorimetry (DSC). Two classes of non-ionic surfactants (sorbitan esters and polyoxyethylene alkyl ethers) were investigated. The results revealed that tuning of cholesterol concentrations can significantly alter the niosome's physical properties including sizes, EE% and membrane fluidity (thermo-responsiveness). The prepared vesicles were in the range of 7.0 ± 1.0 to 14.6 ± 0.8 μm in size. The prepared niosomes showed different abilities to accommodate cholesterol. This was highly dependent on the structure and continuity of the hydrophobic chains of the used surfactants. Span 60-based vesicles containing 30% mol/mol of cholesterol showed the highest EE%. The microstructure and lamellarity of the niosomes were studied using Cryo-SEM. Typical concentric multilayered structures (onion or rose-like) were seen suggesting the formation of multilamellar vesicles. DSC-studies conducted on Span 60-based niosomes containing 30% mol/mol cholesterol revealed liquid-gel transition (T_m and entropy of 43.5 °C and 0.82 kcal/mol, respectively). Such transition reflects potential thermo-responsive properties, which is desirable for ocular delivery.

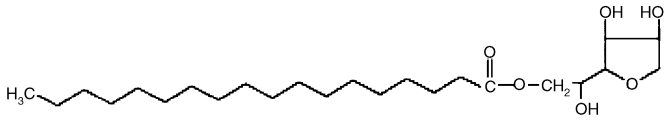
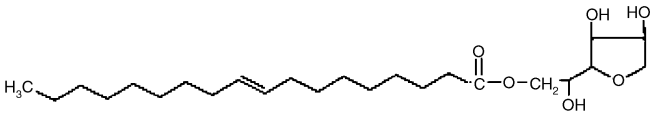
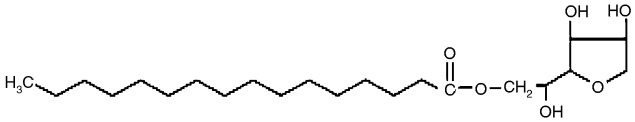
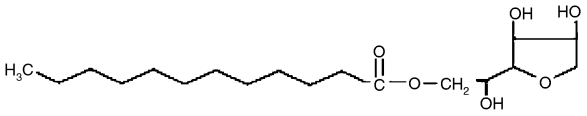
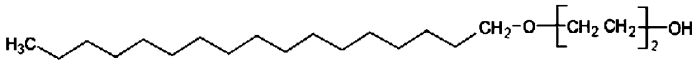
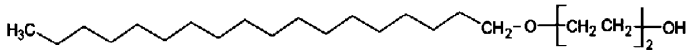
1. Introduction

The cornea of diabetic patients suffers from typical signs and symptoms such as impaired corneal nerve sensation, neurotrophic corneal ulcers and delayed corneal wound repair. These typical signs are known collectively as diabetic keratopathy (Schultz et al. 1981; Aeillo et al. 1998; Frank 2004). Diabetic keratopathy prevalence is 46%–60% amongst diabetic patients (Schultz et al. 1981). The only available treatment options are topical antibiotics and artificial tear substitutes (Mclaughlin et al. 2010). However, these treatments are ineffective because they do not tackle the underlying causes. Therefore the complication is severe and vision loss can ensue (Frank 2004; Mclaughlin et al. 2010).

Naltrexone hydrochloride (NTX) is a potent opioid antagonist. It is widely used for treatment of alcoholism and opioid dependence (Crabtree 1984). Recent reports have demonstrated that topical application of NTX markedly accelerates delayed wound repair of corneas, restore corneal sensation in humans (Zagon et al. 2000), rats (Zagon et al. 1998b) and rabbits (Zagon et al. 1998a), as well as in diabetic rodents (Zagon et al. 2002a; Mclaughlin et al. 2010). The action of NTX is mediated through the blockade of the Opioid Growth Factor (OGF), interaction with the OGF receptor (Zagon et al. 2002b) and consequent enhancement of DNA synthesis and corneal epithelisation. Therefore, ocular application of NTX is thought to be of value in the management of diabetic keratopathy. Whilst there is substantial clinical evidence on the effectiveness of the NTX aqueous solution in diabetic keratopathy, there are hardly any reports on its formulation or incorporation into an appropriate ocular delivery system.

Lipid vesicles prepared from synthetic non-ionic surfactants (niosomes) are considered as a synthetic alternative for the more widely known phospholipid vesicles (liposomes). Niosomes offer many advantages over liposomes. They are more chemically stable and incur a lower production cost. Furthermore, they are potentially biocompatible and biodegradable (Uchegbu and Vyas 1998; Kaur et al. 2004). Over the past two decades, niosomes have been investigated as ocular carriers for a wide range of therapeutic classes such as anti-cholinergics (cyclopentolate), anti-glaucomics (acetazolamide and timolol maleate) and antibiotics (gentamicin). Niosomes significantly improve the ocular bioavailability, via prolonging precorneal drug residence time and minimizing drug loss due to non-productive absorption of the drug via the conjunctiva, nasolacrimal duct and eventually, the GI tract. More interestingly, it has been reported that certain niosomes are devoid of typical ocular irritation signs that are usually associated with other surfactant-based systems (Saettone et al. 1996; Vyas et al. 1998; Aggarwal et al. 2004; Abdelbary and El-Gendy 2008).

Table 1: Chemical structure, phase transition temperature, HLB and vesicle-forming ability of the used surfactants

Names and chemical structutre of surfactants	TC[a] (C°)	HLB[b]	Vesicle formation without cholesteral*	Minimum level of cholesterol required for vesicle formation (mole%)*
Sorbitan monostearate (Span 60) 	53–57	4.7	yes	–
Sorbitan monooleate (Span 80) 	<–30	4.3	no	20
Sorbitan monopalmitate (Span 40) 	43–48	6.7	yes	–
Sorbitan monolaurate (Span 20) 	< 10	8.6	no	10
Polyoxyethylene 2 stearyl ether (Brij 72) 	40–43	4.9	yes	–
Polyoxyethylene 2 cetyl ether (Brij 52) 	36–38	5.3	yes	–

* Vesicle formation was determined by maltese cross formation when viewed using a polarized light microscope.
[a] Phase transition temperature of the unhydrated samples.
[b] HLB values were given by suppliers.

Many classes of non-ionic surfactants have been utilized as niosome-forming lipids, most notably, sorbitan esters (Spans™) and polysorbates (Tweens™). This is most probably due to their complete biodegradability, biocompatibility and minimal ocular side effects (Abdelbary and El-Gendy 2008; Saettone et al. 1996; Alany et al. 2006).
Vesicles should have certain attributes in relation to their potential use as vehicles for ocular drug delivery. These include:

- Size of the vesicle which should be large enough to resist drainage by reflex tear production or eye blinking. Fitzgerald et al. (1987b) studied the effect of vesicle's size on their precorneal retention time. They reported that large multilamellar vesicles were more promising than smaller unilamellar ones due to their higher drug entrapment efficiency and slower drainage (Fitzgerald et al. 1987a; Hathout et al. 2007).
- Shape of the prepared niosomes which should ideally show some irregularities to properly fit into the cul-de-sac and lodge on the eye surface (Uchegbu and Vyas 1998; Uchegbu et al. 1997; Uchegbu et al. 1992),
- Thermo-responsiveness, which is desirable for controlled drug release in a timely manner. This is particularly useful for the noisomes to avoid being flushed by blinking and nasolacrimal drainage (Uchegbu et al. 1997; Uchegbu and Vyas 1998).

The abovementioned criteria were typically attributed to discomes and polyhedral niosomes which are modified vesicular structures and as such different from conventional niosomes.
Discomes were prepared by hydrating an optimum ratio of lipid mixture comprising hexadecyl diglycerol and cholesteryl poly-24-oxyethylene ether commercially known as Solulan C24 (Uchegbu et al. 1992). Polyhedral niosomes were formed by incubating the preformed niosomal dispersion with Solulan C24 at 75 °C for 1 h. This resulted in large (11–60 μm) and multifaceted vesicular systems (Uchegbu et al. 1992). Since then, only one study was carried out by on discomes for ocular drug deliv-

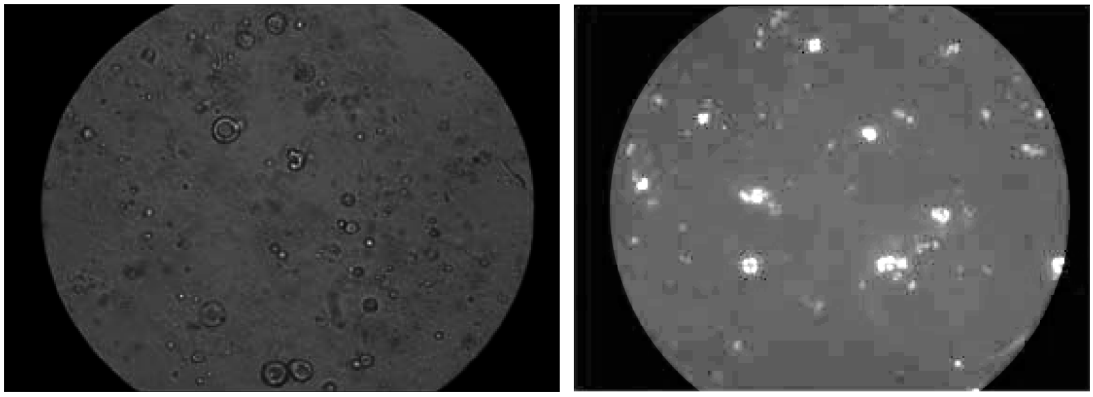


Fig. 1: Representative micrographs of Brij 72:cholesterol 7:3 mol/mol niosomes viewed under plane (left) and polarized light (right)

ery of timolol maleate (Vyas et al. 1998). One possible reason for such scarcity is the need for a relatively high temperature during discome preparation which may affect the chemical stability of some thermo-labile therapeutic agents. Furthermore, spherical niosomes possess more stable membranes than polyhedral ones due to the presence of cholesterol as part of their structure (Arunothayanun et al. 1999).

Cholesterol is an essential membrane stabilizer commonly found in niosomes. Cholesterol cannot form bilayer membrane vesicles alone, due to its unique structural features and geometrical packing properties, poor water solubility and extreme hydrophobicity (Israelachvili et al. 1980). However, cholesterol can stabilize the membrane of the vesicles, alter their size, abolish phase transition of lipid vesicles, decrease membrane permeability, and alter membrane fluidity (Uchegbu and Florence 1995). Nevertheless, there is some controversy in relation to the effect of cholesterol on niosome's physical properties such as entrapment efficiency and the optimum level of cholesterol which is required for more efficient drug delivery to the eye (Uchegbu and Florence 1995). This study is an attempt to optimize cholesterol level for NTX niosomes intended for topical ocular administration. More specific objectives include the prepararion of conventional NTX ocular niosomes that fulfil the abovementioned criteria with respect to vesicle size, drug entrapment efficiency, morphology and thermo-responsiveness.

2. Investigations, results and discussion

The following surfactants: sorbitan esters (Span 20, Span 40, Span 60 and Span 80) and polyoxyethylene alkyl ethers (Brij 52 and Brij 72) have been investigated for their ability to form niosomes with different concentration of cholesterol (0% to 50% mol/mol) using the thin film hydration method. Table 1 shows the chemical structure, gel-liquid transition (T_m) temperature, hydrophilic-lipophilic balance (HLB) of the used surfactants and summarizes the ability of each surfactant to form niosomes on its own and the minim level of cholesterol required to form niosomes. The formed niosomes were visualised under a polarized light microscope (Fig. 1).

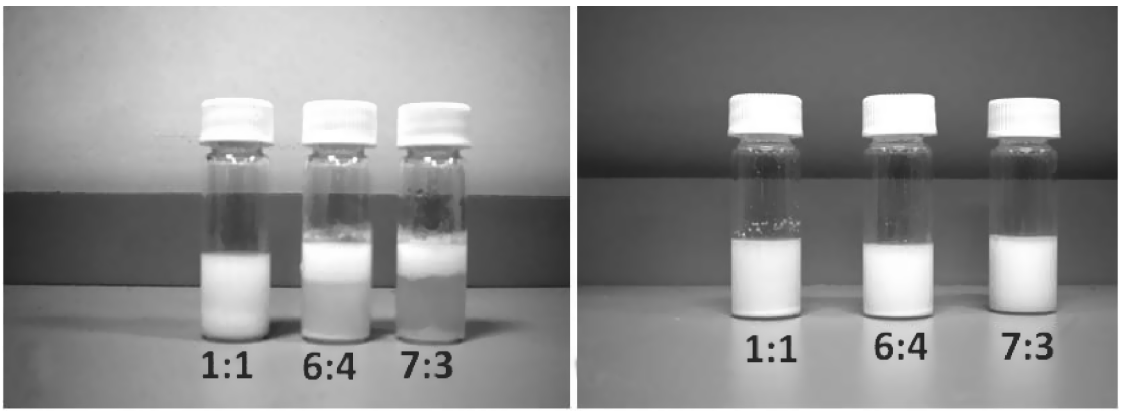
Span 40, Span 60, Brij 52, and Brij 72 formed surfactant vesicles with 0% cholesterol. On the other hand, Span 20 and Span 80 could not self-assembled into niosomes unless 10% to 20% mol/mol) of cholesterol was incorporated. These findings could be attributed to the geometrical packing parameters of the used surfactants. The geometrical packing parameters depend on the optimal polar head group area $\mathbf{a_o}$, the volume **v** of the hydrocarbon chains and the maximum effective length that the hydrocarbon chains can assume (critical chain length) $\mathbf{l_c}$. Accordingly, the value of the dimensionless 'packing parameter' $\mathbf{v/\ a_o\ l_c}$ will determine whether a certain amphiphile or polar lipid will form niosomes ($1/2 < \mathbf{v/\ a_o\ l_c} < 1$), or other surfactant aggregates such as spherical micelles ($\mathbf{v/\ a_o\ l_c} < 1/3$), non-spherical micelles ($1/3 < \mathbf{v/\ a_o\ l_c} < 1/2$), or inverted micellar structures ($\mathbf{v/\ a_o\ l_c} > 1$) or even precipitate out of solution (Israelachvili et al. 1980; Israelachvili and Mitchell 1975).

Niosomes containing 0% cholesterol gelled extensively upon cooling down to room temperature. The formed gel was difficult to separate from the non-entrapped drug. Systems prepared using Brij 52 showed phase separation and creaming upon storage overnight when formulated using a relatively higher percentage of cholesterol (30%, 40% and 50 mol %), as shown in Fig. 2A. These findings suggest that the abovementioned formulations are not suitable for further investigation as potential ocular delivery systems for NTX.

The mean volume diameters of the prepared vesicles ranged from 7.0 ± 1.0 to $14.6 \pm 0.8\ \mu m$ (Table 2). The size of the prepared niosomes increased with increasing cholesterol concentration. However, this effect was insignificant (ANOVA, $P > 0.05$) due to the broad size distribution and heterogeneity of the niosomes prepared using the thin film hydration method (Hope et al. 1986). Cholesterol is a rigid molecule with a truncated cone shape. It can be intercalated between the fluid hydrocarbon chains of the amphiphile when hydrated at temperature above the gel-liquid transition temperature. It can also results in largersvesicles. However, the ability of the investigated amphiphiles to accommodate cholesterol were obviously decreased when cooled down below the melting point, where the hydrocarbon chains are fully extended (all-trans) and exist in a gel state. Fig. 2B shows plain niosomal dispersions containing different levels of cholesterol after storage at room temperature overnight. Such storage allows for the vesicle's membrane to anneal and correct any membrane defect. Niosomal dispersions, containing cholesterol >30% mol/mol, were poorly hydrated and excess cholesterol precipitated out which may be attributed to the compromised ability of the hydrocarbon chains of the used surfactant to accommodate cholesterol (Israelachvili et al. 1980; Grant et al. 2001).

The results show that the size of the prepared vesicles was significantly (ANOVA, $P < 0.05$) dependent on the HLB of the used surfactants. For instance, the sizes of Span 60-based niosomes were significantly smaller than the Span 40-based ones (Table 2). Lipid aggregation and self-assembly in aqueous medium is mainly controlled by two opposing electrostatic forces, namely, the hydrophobic attraction forces between the hydrocarbon tail chains and the electrostatic repulsion of the polar head groups (Tanford 1973). Surface free energy increases with any decrease in hydrophobicity (Yoshioka et al. 1994). Hence, less hydrophobic (higher HLB) surfactants should yield larger vesicles.

Table 2 shows the entrapment efficiency of NTX in the prepared niosomes expressed as percentage (EE %) which ranged



(A) (B)

Fig. 2: Niosomal dispersions of different Brij 52: cholesterol (A) and Span 60: cholesterol molar ratios (B), showing: (A) Phase separation (B) Poor hydration at surfactant: cholesterol ratios 6:4 and 1:1

Table 2: Effect of surfactant type and cholesterol level and on the vesicle size and entrapment efficiency (EE %) of the prepared niosomes. Results are presented as mean ± SD, n = 3

	Cholesterol level (% mole/mole)			Cholesterol level (% mole/mole)		
	30%	40%	50%	30%	40%	50%
	Vesicle sizes (μm)			EE%		
Span 20	14.2 ± 0.6	14.5 ± 0.65	14.6 ± 0.8	3.5 ± 0.23	3.0 ± 0.15	2.5 ± 0.23
Span 40	12.7 ± 1.0	13.0 ± 1.5	13.4 ± 1.2	9.0 ± 0.67	7.0 ± 0.62	6.0 ± 0.54
Span 60	8.3 ± 0.62	8.4 ± 0.75	8.5 ± 0.90	13.5 ± 0.56	9.0 ± 0.45	7.0 ± 0.67
Span 80	7.0 ± 1.0	7.4 ± 0.88	7.5 ± 1.20	3.2 ± 0.22	4.0 ± 0.35	2.8 ± 0.25
Brij 72	9.0 ± 0.82	9.5 ± 0.88	9.8 ± 1.0	7.0 ± 0.55	5.0 ± 0.47	4.5 ± 0.32

from 2.5 ± 0.23 to 13.5 ± 0.56%. Span 40 and Span 60 showed the highest EE%, whereas Span 20 and Span 80 showed the lowest EE% of NTX. The main factor affecting the entrapment of small water soluble drug molecules in lipid vesicles is the permeability of the biomolecular membranes and the structure continuity of the hydrocarbon chain of the bilayer forming surfactant (Grant et al. 2001). Span 40 and Span 60 have saturated acyl chains [palmityl (C-16) and stearyl (C-18) chains respectively] which are in a semi-solid gel state at normal ambient conditions (Grant et al. 2001). Such features render these vesicles less leaky to water soluble drug molecules. On the contrary, Span 20 and Span 80 are fluid at room temperature as well as in the presence of cholesterol i.e are disorganized due to the trans-gauche conformations of their acyl chains.. The kinks formed in the acyl chain of Span 80 are due to the double bond. These kinks create vacancies in the hydrophobic chains. This renders Span 80-based vesicles more leaky and permeable to NTX than the Span 20-based ones. This explains why Span 80-based niosomes showed the lowest EE% of NTX. Furthermore, the presence of the double bond as part of the Span 80 structure necessitates the need for higher amount of cholesterol (40%) compared with that required by all the other surfactants under investigation. The entrapment efficiency was in the following order: Span 60 > Span 40 > Brij 72 > Span 20 > Span 80. These results support the hypothesis that the higher the transition temperature of the surfactant, the higher the corresponding EE% for water soluble solutes (Yoshioka et al. 1994).

Moreover, it was found that the cholesterol level significantly (ANOVA, $P < 0.05$) affects the ability of niosomes to entrap the water soluble drug, NTX. Fig. 3 illustrates the effect of cholesterol levels on the EE%. Increasing cholesterol concentration can improve EE% of NTX, but only to a certain extent. Any further increase of cholesterol beyond a certain concentration (30% mol/mol for Span 40, and Span 60 and 40% mol/mol for Span 80) markedly decreases EE%. The initial increase in EE% of NTX is due to the initial intercalation of cholesterol molecules between the hydrocarbon chains of the surfactant bilayers, hence, the decrease in permeability to the water soluble molecule, NTX. Further increase in cholesterol concentration increases the rigidity of the bilayer membranes by virtue of cholesterol's rigid structure and characteristic inverted truncated cone shape (Israelachvili et al. 1980). This results in disruption of the vesicles' bilayer structure which compromises their ability to entrap NTX (Manosroi et al. 2003). This can lead to a decrease in the total amount of lipid available for encapsulating the drug. Consequently, there is a decrease in the ability of niosomes to entrap such water soluble molecules. These findings are manifested in Fig. 2B, which shows precipitate at a cholesterol level higher than 30 mol%. These results are in good agreement with previous reports where uncharged nio-

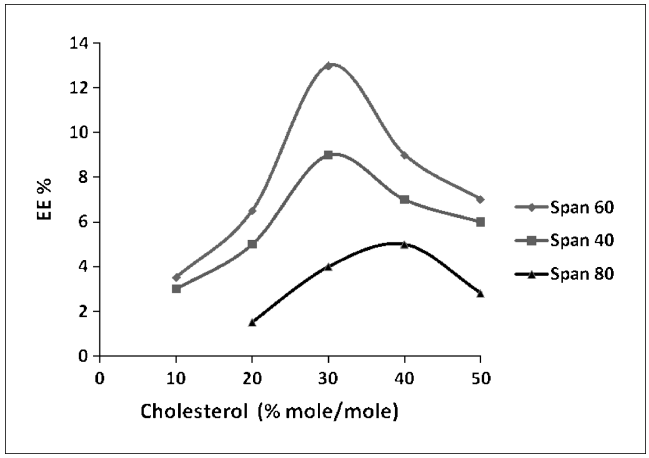


Fig. 3: Effect of cholesterol concentration on NTX entrapment efficiency expressed as percentage of the prepared niosomes (EE%)

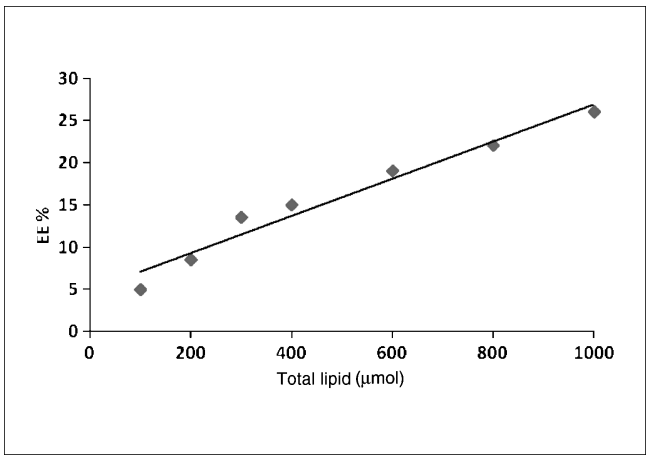


Fig. 4: Effect of total lipid content on NTX entrapment efficiency (EE%) in Span 60: cholesterol 7:3 niosomes

somes containing 1:0.5 mol/mol Tween 60 and cholesterol had higher EE% than those containing Tween 60 and cholesterol at molar ratio of 1:1 (Abdelbary and El-Gendy 2008).

The effect of total lipid content on the EE% of NTX was studied and the results are shown in Fig. 4. The results reveal a linear increase ($R^2 > 0.96$) in the EE% of the drug with increasing the total lipid available for hydration. Any increase of drug to total lipid ratio is associated with an increase in the percentage of drug to be encapsulated.

Microstructure and lamellarity of the prepared vesicles were studied using Cryo-SEM. The captured micrographs reveals spherical-shaped niosomes with smooth surfaces and typical folded multilayers organised in an onion-like or rose-like morphology indicating multilamellarity of the prepared vesicles. It is worth noting that Cryo-SEM has been shown to be more reliable than freeze-fracture transmission electron microscopy (FF-TEM) (Egelhaaf et al. 2003; Perrie et al. 2007). Steps involved in FF-TEM are likely to promote the formation of artefacts during the sample preparation and replica creation (Egelhaaf et al. 2003; Perrie et al. 2007).

DSC was used to study the interaction of cholesterol, drugs, and proteins with phospholipid-forming vesicles (liposomes) (El Maghraby et al. 2005; Lo and Rahman 1995; Papahadjopoulos et al. 1975; Rolland et al. 1991). In this study, DSC was employed to understand the interaction of cholesterol with the single-chain non-ionic surfactant and monitor its effect on the gel/liquid transition of the prepared vesicles. Alkyl chains are traditionally fluid and highly permeable to small molecules above their

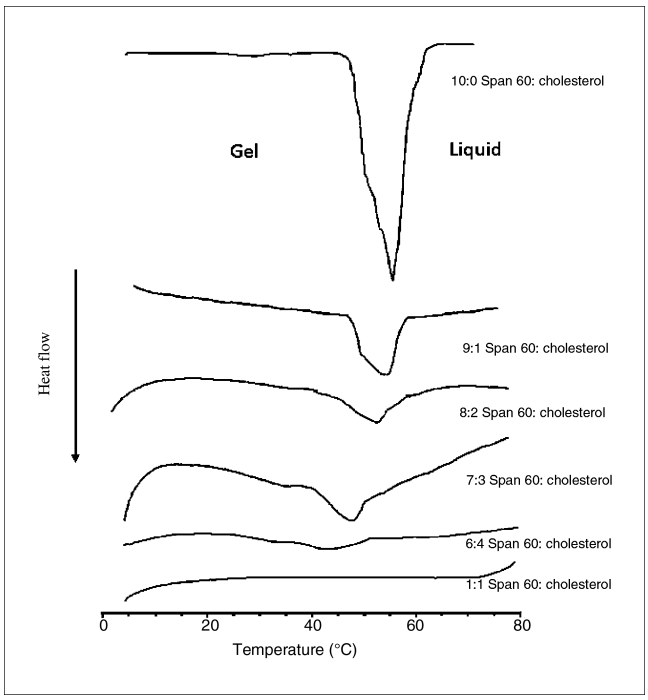


Fig. 6: DSC thermograms of Span 60-based niosomes containing different concentration of cholesterol

gel/liquid transition temperature. This is due to the probable disorganization or trans-gauche conformation along the hydrocarbon chains. When a surfactant cools down below its transition temperature, hydrocarbon chains become more organized fully extended (all-trans), and form impermeable gels (Israelachvili et al. 1980). Fig. 6 demonstrates the effect of cholesterol level on the gel-liquid transition of Span 60.

Table 3 presents two parameters, the transition temperature, T_m, which is the temperature peak point at which transition (melting) is half-complete and the transition enthalpy which is the amount of heat in kilocalories required for the entire transition to take place. The thermogram of Span 60-based vesicles with 0% cholesterol shows a thermal event at 63 °C which is markedly higher than that of the constituting Span 60 surfactant (MP = 55 °C) in the unhydrated form. This increase in the T_m of the Span 60 may be attributed to the hydration and self-assembly of the surfactant molecules forming closed bilayer structures in water. As such, a higher energy is required to break the strong hydrophobic interaction between surfactant moieties in the hydrated state. Cholesterol has a marked effect in decreas-

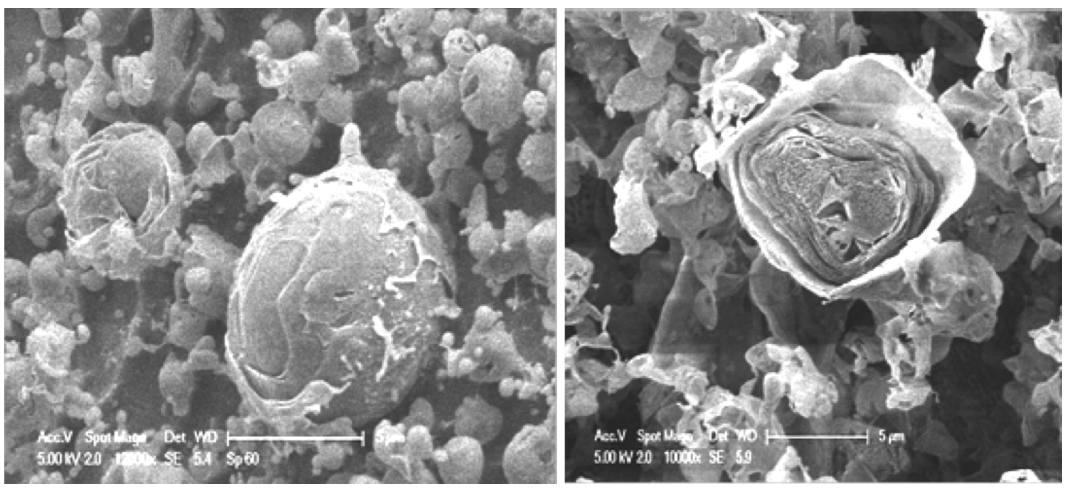


Fig. 5: Cryo-SEM micrographs of Span 60-based (left) and Brij 72-based (right) niosomes

Table 3: DSC parameters of Span 60-based noisome dispersions containing different concentration of cholesterol

Cholesterol concentration (% mol/mol)	T_m (°C)	Enthalpy (Cal/mol)
0	63.44	22.98
10	55.00	3.10
20	51.00	2.05
30	47.22	0.82
40	43.50	0.43
50	No transition	–

ing both T_m and transition enthalpy. This is clearly evident by the complete disappearance of the gel-liquid transition of Span 60 at 50% mol/mol. This is possibly due to intercalation of cholesterol molecules between the lipid bilayer and subsequent decrease in membrane fluidity. These findings are in agreement with what has been previously reported (Taylor and Craig 2003). However; unlike phospholipids, no pre-transition peak was seen with Span 60 in Fig. 6. This is due to the well defined structure and composition of the used non-ionic surfactant. Finally, it is worth noting that the residual gel-liquid transition noticed with 30% and 40% mol of cholesterol may be advantageous. Such transition is expected to impart thermo-responsiveness to the prepared niosomes and as such render them promising carriers for ocular drug delivery. On the contrary, complete abolishment of the gel-liquid transition makes such niosomes rigid and as such less attractive for ocular applications.

In conclusion, the cholesterol level can significantly alter the physical properties of the prepared NTX niosomes; most notably, their thermo-responsiveness. Such behaviour is likely to render these vesicles more amenable to the ocular temperature and less likely to induce a foreign body sensation (and subsequent reflex tear production and blinking) when instilled topically to the eye surface. These niosomes will be the subject of future research aimed at delivering NTX topically to the surface of the eye with particular emphasis on the management of diabetic keratopathy.

3. Experimental

3.1. Materials

NTX was purchased from Mallinckrodt Inc (St. Louis, MO, USA). Span 20, Span 40, Span 60, Span 80, Brij 52, Brij 72 and cholesterol were purchased from Sigma-Aldrich, St. Louis, USA. All other solvent and buffer salts are of analytical grade and were used as received.

3.2. Methods

3.2.1. Niosme's preparation using thin film hydration

Niosomes were prepared by adopting the procedure of Azmin et al. (1985) Briefly, a specified amount (300 μmol) of lipids (surfactant:cholesterol) at a molar ratio of 1:0, 9:1, 8:2, 7:3, 6:4, and 5:5 was dissolved in a chloroform: methanol (2:1 v/v) mixture, and rotary evaporated (Heidolph, Laborota 4000, GmbH, Germany) to form a thin film. The dried lipid film was purged with a stream of nitrogen for 5 min in order to get rid of residual traces of the organic solvent. The lipid film was then hydrated with either 6 ml of phosphate buffer saline (PBS) or NTX solution (1 mg/ml) and swirl-evaporated at 200 rpm for 2 h. The resultant niosomal suspension was set aside for at least 2 h at room temperature to allow for the vesicle's membrane to anneal. The formed niosomes were kept in a fridge for subsequent analyses.

3.2.2. Plane and polarized light microscopy

To assure vesicle formation, all lipid dispersions were examined using a light microscope equipped with a cross-polarizer (Leica DMR, GmbH, Germany). Briefly, a drop of the prepared lipid dispersion was placed on a microscope g slide, covered with a glass cover slip and examined using a polarized light microscope. The samples were examined for phase separation, vesicle, and crystal formation. Large multilamellar vesicles were recognized by their characteristic multi-layer and Maltese cross texture (Manosroi et al. 2003).

3.2.3. Cryogenic scanning electron microscopy (Cryo-SEM)

A small drop of the prepared niosomal dispersion was sandwiched between two fracture rivets (internal diameter = 1.58 mm) that were fixed on a gold-plated copper sample holder. The whole assembly was connected to a transfer rod and then plunged into liquid nitrogen at −190 °C under vacuum and transferred into a Cryo-unit (Gattan Alto 2500, England) which includes a fracture stage and a sputter coater operating at a coating temperature of less than −120 °C. A fracture was created by removing the upper rivet using a scalpel. The fractured sample was heated up to −85 °C for 30 min to sublime the surface moisture. Subsequently, the sample was gold coated and transferred to the SEM's cryo-chamber (Philips XL30S FEG, Netherlands) for imaging at −140 °C.

3.2.4. Entrapment efficiency (EE %) of naltrexone hydrochloride

The lipid vesicles were separated from the non-entrapped drug by diluting 2 ml of niosome dispersion to 30 ml with PBS (297 mOsm/kg) prior to centrifugation at 60,000 g (Sorvall Discovery 100S, USA) for 30 min. The formed niosomal pellets were washed with additional 30 ml of PBS and centrifuged for further 30 min. The niosomal pellets were re-suspended in 2 ml PBS and isopropanol was added (1:1 v/v) followed by 5 ml of PBS. The dispersion was re-centrifuged and the supernatant was quantitatively assayed for NTX content using high performance liquid chromatography (HPLC). The used HPLC system (Agilent 1200, Germany) was equipped with a C18-reverse phase column (Supelcosil LC-18DB 25 cm x 4.6 mm, 5 μm,). The mobile phase comprised acetonitrile:phosphate buffer, 40 mM, pH 4, and detection was achieved using a diode array detector at 214 nm. The drug entrapment efficiency expressed as percentage (EE %) was calculated using the following equation:

$$EE\% = \frac{A}{Ao} \times 100$$

Where A is the amount of NTX entrapped in niosomes and A_o is the initial amount of NTX used.

3.2.5. Vesicle size measurement

Sizes of the prepared vesicles were determined by a laser diffraction instrument (Mastersizer 2000, Malvern Instruments, UK). The samples were properly diluted with PBS and measured at 25 °C. The sizes were expressed as mean volume diameter (D4,3). The experiments were done in triplicate.

3.2.6. Differential scanning calorimetry

Niosomal dispersions containing 100 mg total lipid/ml were prepared by the thin-film hydration method. The niosomal dispersions comprised 1:0, 9:1, 8:2, 7:3, 6:4, and 1:1 Span 60:cholesterol molar ratio. A small amount (approximately 5 mg) of the prepared niosomes was accurately weighed in an aluminium pan, covered with an aluminium lid and hermitically sealed using a pan press (Thermal Science, USA). Another pan containing an equivalent amount of water was sealed and used as a reference cell. The temperature of the pans was raised from 4 to 80 °C, at a rate of 5 °C/min using a differential scanning calorimeter (Q 1000, Tzero series, Thermal Analysis, USA). Nitrogen gas was purged at flow rate 45 ml/min.

3.2.7. Statistics

To compare the mean values of the vesicle size and EE% of the prepared niosomes and to assess statistical significance, a one-way analysis of variance (ANOVA) was performed at a 5% significance level, using GraphPad Software Version 3.05, San Diego California, USA.

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